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Biocatalytic Carbene Transfer Using Diazirines

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Abstract

Biocatalytic carbene transfer from diazo compounds is a versatile strategy in asymmetric synthesis. However, the limited pool of stable diazo compounds constrains the variety of accessible products. To overcome this restriction, we have engineered variants of *Aeropyrum pernix* protoglobin (*Ape*Pgb) that use diazirines as carbene precursors. While the enhanced stability of diazirines relative to their diazo isomers enables access to a diverse array of carbenes, they have previously resisted catalytic activation. Our engineered *Ape*Pgb variants represent the first example of catalysts for selective carbene transfer from these species at room temperature. The structure of an *Ape*Pgb variant, determined by microcrystal electron diffraction (MicroED), reveals that evolution has enhanced access to the heme active site to facilitate this new-to-nature catalysis. Using readily prepared aryl diazirines as model substrates, we demonstrate the application of these highly-stable carbene precursors in biocatalytic cyclopropanation, N–H insertion, and Si–H insertion reactions.

Graphical Abstract

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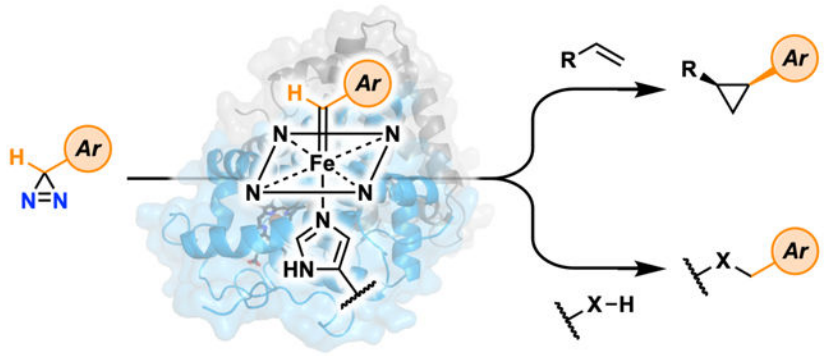
Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supplementary figures, materials, experimental methods, compound synthesis and characterization, HPLC and GC-FID calibration curves, HPLC and SFC traces (PDF)

The coordinates and structure factors for the structure of ApePgb GLVRSQL has been deposited to the PDB under accession code 7UTE. The corresponding density map has been deposited to the EMDB under code 26768.



Keywords

biocatalysis; carbene transfer; diazirines; asymmetric catalysis; MicroED; protoglobin

Over the past decades, biocatalysis has enabled the efficient and sustainable production of valuable compounds by drawing on the phenomenal activity and selectivity of enzymes.[1] Among the surge of novel biotransformations implemented in the past few years, hemoprotein-catalyzed carbene transfer reactions[2] have emerged as powerful new-to-nature transformations (Scheme 1a), including cyclopropanation,[3] bicyclobutanation,[4] and C–C bond formation.[5] Though these enzymes yield many types of products, their synthetic potential has been limited by the small range of accessible carbenes. All efforts on these systems to date have used diazo compounds such as ethyl diazoacetate (EDA), which require the presence of an electron-with-drawing group (EWG) adjacent to the diazo moiety to be safely manipulated.[6] In the absence of such substituents the diazo reagent is unstable and potentially explosive, making the corresponding carbenes unattractive for use at scale. More stable carbene precursors would allow access to a wider variety of iron carbenoid intermediates and promote preparative and industrial applications.

Diazirines are cyclic isomers of diazo compounds and are used as probes in photoaffinity labeling and chemical proteomics (Scheme 1b).[7–9] These well-studied carbene precursors are more stable than the corresponding diazo compounds and, as a result, do not require an EWG substituent to be handled safely. The tradeoff of increased stability is that more forcing conditions (high temperatures or UV photolysis, e.g.) are required for diazirine activation,[10] resulting in low yields or poor selectivities in synthetic applications.[11,12] Main group Lewis acids have been shown to activate diazirines, albeit with limited synthetic utility.[13] Meanwhile, in contrast to the well-explored transition metal-catalyzed carbene transfer from diazo compounds,[14] the few examples of analogous diazirine-derived metallocarbenes known to form at room temperature have never demonstrated productive catalytic carbene transfer.[15] This is unfortunate as diverse diazirines are readily made via numerous synthetic routes,[16–18] including the recent single-step conversion of α-amino acids to 3*H*-diazirines.[19] The development of biocatalysts capable of using diazirines as carbene precursors could dramatically expand the scope of carbenes accessible in enzymatic transformations. In this work we introduce such a biocatalyst (Scheme 1c), demonstrating

for the first time that stable diazirines can be biocatalytically activated to a carbene under mild conditions and productively transferred in multiple types of reactions.

Given the isomeric relationship between diazirines and diazo compounds, we began by assessing whether a hemoprotein might also accept a diazirine as a substrate. We first tested whether a *Rhodothermus marinus* cytochrome *c* variant that effects B–H insertion into borane **1** using diazo compound **2** as a carbene donor (Figure 1a)[20] could utilize the isomeric diazirine **4** to access the same putative iron-carbenoid intermediate and yield organoborane **3**. Although the cytochrome *c* variant displayed no activity for this transformation, a control reaction containing free heme alone exhibited trace levels of product formation (Table S1). Emboldened by this observation, we screened a collection of engineered heme proteins for the same B–H insertion reaction using diazirine **4** as the carbene source. Gratifyingly, *Aeropyrum pernix* protoglobin variant Y60G F145Q (*Ape*Pgb GQ) exhibited formation of the organoborane product **3** in 0.9 % yield in a whole *E. coli* cell context, providing a reasonable starting point to assess reaction evolvability (Figure 1b). This enzyme was previously engineered for the cyclopropanation of unactivated alkenes using EDA.[21] *Ape*Pgb is an attractive platform for enzyme engineering because its high thermostability[22] allows it to accept activating mutations that are often destabilizing.[23] Additionally, its high expression level in *Escherichia coli* (>200 mg/L culture) means that protoglobin-based biocatalysts can be produced with ease and at low cost. Following validation of this activity with purified enzyme, amino acid positions near the enzyme active site were subjected to site-saturation mutagenesis (SSM) and the resultant libraries were screened for improved B–H insertion activity (Figure 1c). Iterative screening of these sites yielded a new variant, *Ape*Pgb W59L Y60V F145Q (*Ape*Pgb LVQ), that exhibited a nearly three-fold increase in organoborane yield. Notably, the mutations had a pronounced effect on enzyme activity only when using diazirine **4** as a carbene source, as yield of **3** with diazo compound **2** exhibited little change across these variants (Figure S1). This confirms that enzymatic activation of a diazirine can be enhanced by directed evolution.

Having demonstrated that carbene transfer from diazirine **4** was both enzyme-catalyzed and amenable to evolution, we then set out to pursue the primary objective: selective enzymatic transfer of a carbene bearing no EWG substituent. We decided to pursue 3-phenyl-3*H*-diazirine **6a** due to its similarity to **4** and its ease of synthesis. Notably, the corresponding diazo isomer, phenyldiazomethane, is known to be unstable and explosive.[6] Successful carbene transfer from **6a** would enable safe biocatalytic access to the phenyl carbene. However, the use of a mono-substituted diazirine removed stereochemistry in the B–H insertion product. Thus, to assess the selectivity conferred by the enzyme, we shifted focus to a carbene transfer activity yielding a chiral product. Using **6a** as a carbene source, *Ape*Pgb LVQ catalyzed the cyclopropanation of acrylate **5** to give chiral cyclopropane **7a** at <1% yield with 2:1 *cis*:*trans* diastereoselectivity. Free heme also catalyzed this transformation, albeit at a much lower level, and heavily favored production of *trans*-**7a**. Thus, we targeted production of the higher energy diastereomer *cis*-**7a** for directed evolution of the enzyme.

*Ape*Pgb variant libraries made by SSM and random mutagenesis were screened for enhanced production of the desired *cis*-cyclopropane. Five sequential rounds of directed

evolution resulted in variant *Ape*Pgb GLAVRSQLL bearing mutations C45G, W59L, Y60A, G61V, V63R, C102S, F145Q, I149L, and F175L relative to wild-type *Ape*Pgb. This variant exhibited >150-fold improvement in *cis*-**7a** formation in whole *E. coli* cells relative to *Ape*Pgb LVQ, producing **7a** in 28% yield with 6:1 *cis*:*trans* diastereomeric ratio (dr) and 86:14 enantiomeric ratio (er) for the *cis*- isomer (Figure 2). Using optimized conditions developed with purified enzyme resulted in 53% yield with 6.9:1 *cis*:*trans* dr and 80:20 er (Figure 3a).

Having reached good activity for the cyclopropanation reaction, we were curious how substitutions on the aryl ring of the diazirine are accommodated by the enzyme. To this end, *p*-fluoro- (**6b**), *p*-chloro- (**6c**), and *p*-methoxyphenyl-3*H*-diazirine (**6d**) were synthesized and used in activity assays. Diazirine **6b** gave 69% yield of cyclopropane **7b** with 8.5:1 *cis*:*trans* dr and 88:12 er, and **6c** gave 64% yield of cyclopropane **7c** with 9.6:1 *cis*:*trans* dr and 81:19 er. (Figure 3a). No product formation was detected by GC-MS using **6d**, suggesting that the presence of either electron-donating or bulky substituents on the ring impedes binding or catalysis. Additionally, 3-benzyl-3*H*-diazirine **6e** was tested to assess activity with an alkyl diazirine. No product was observed by GC-MS, indicating that deeper understanding of enzymatic diazirine activation may be required to access carbenes from such diazirines.

We next assessed the ability of *Ape*Pgb GLAVRSQLL to catalyze other carbene transfer reactions (Figure 3b). Using **6a** as a carbene source, the enzyme cyclopropanated styrene **8**, affording 1,2-diphenylcyclopropane **9** in 61% yield with 12:1 *cis*:*trans* dr. We also tested for enzymatic C–X bond formation, using aniline **10**, silane **12**, and anisole **14** as substrates to assess N–H, Si–H, and C–H insertion activities, respectively. *Ape*Pgb GLAVRSQLL produced the N–H insertion product **11** in 15% yield and Si–H insertion product **13** in 5% yield. However, none of the corresponding C–H insertion product was observed by GC-MS. The formation of **11** and **13** by *Ape*Pgb GLAVRSQLL illustrates that this enzyme can serve as a good starting point for the evolution of variants catalyzing such X–H insertion reactions.

Taken together, the accommodation of different diazirines and substrates, including those for unoptimized activities, highlights the functional flexibility of the *Ape*Pgb enzyme scaffold. To interrogate the functional changes introduced through evolution, we determined the first structure of an *Ape*Pgb variant, GLVRSQL, by microcrystal electron diffraction (MicroED). In general, the protoglobin fold is maintained with an overall r.m.s.d. of <0.8 Å across backbone atoms in comparison to structures of *M. acetivorans* protoglobin (76% sequence similarity), the only homolog whose structure has been reported.[24,25] Additionally, the porphyrin ring of the heme cofactor exhibits the ruffled distortion present in other protoglobin structures.[26] However, residues 60–70, which adopt a rigid helical conformation in the homologous protein, appear to be restructured as a loop in the MicroED structure of *Ape*Pgb GLVRSQL (Figure 4). This region contains the mutation V63R, the first mutation introduced during evolution for cyclopropanation chemistry, which resulted in a 14-fold boost in product yield. This boost reasonably stems from the unwinding of the rigid helix into a more flexible/dynamic loop, which creates a larger cavity around the enzyme active site and likely facilitates access to the heme iron. Two mutations subsequently introduced into this region, V60A and G61V, contributed to an additional 4-fold boost in cyclopropane yield. This structure-function analysis, further elaborated in the Supplementary Information,

suggests that rearrangement of the polypeptide backbone around the newly formed active site of *Ape*Pgb enhances enzymatic diazirine activation.

Given the mild conditions required for this enzymatic activity, we sought to further interrogate the chemical details underlying this catalysis. During condition optimization with purified protein, control experiments demonstrated that ambient light is not required for diazirine activation as reactions run under strictly dark conditions exhibited comparable yield (Figure S2). We hypothesized that the enzyme may isomerize the diazirine to the corresponding diazo *in situ*, then perform carbene transfer in a manner similar to that previously demonstrated by other heme enzymes. To test this hypothesis, we used dibenzocyclooctyne amine **S1** (DBCO-NH_2) as a probe for the diazo intermediate. In the presence of a diazo compound, the DBCO-NH_2 alkyne undergoes a rapid [3 + 2] cycloaddition to afford the pyrazole product **S2** (Figure S3a).[27] Intriguingly, when DBCO-NH_2 was added to a sample of *Ape*Pgb GLAVRSQLL in the presence of **6a**, the pyrazole product was observed at a higher level than for either the same concentration of free heme or heat-denatured enzyme (Figure S3b). Since the concentration of pyrazole product **S2** is directly related to the amount of diazo compound in solution,[27] this is consistent with enzymatic isomerization of diazirine **6a** to the corresponding diazo isomer followed by capture by DBCO-NH_2. This suggests that ring opening is a potential mechanism for diazirine activation by *Ape*Pgb GLAVRSQLL.

In conclusion, this work presents the first example of catalytic diazirine activation and subsequent selective carbene transfer; this new-to-nature chemistry is accomplished enzymatically under mild aqueous conditions and requires no exogenous heat or light. We believe that the enzymatic platform will open a whole new set of transformations accessible by biocatalytic carbene transfer. For example, access to arylcarbenes, as shown here, would otherwise require hazardous diazo compounds. Encouraged by these results, we envision that further engineering of protoglobin variants could provide access to even more challenging species, such as alkylcarbenes, and provide access to more X–H insertion reactions based on the nascent activities presented here. The potential synthetic scope achievable with diazirines is both exciting and promising for the advancement of biocatalysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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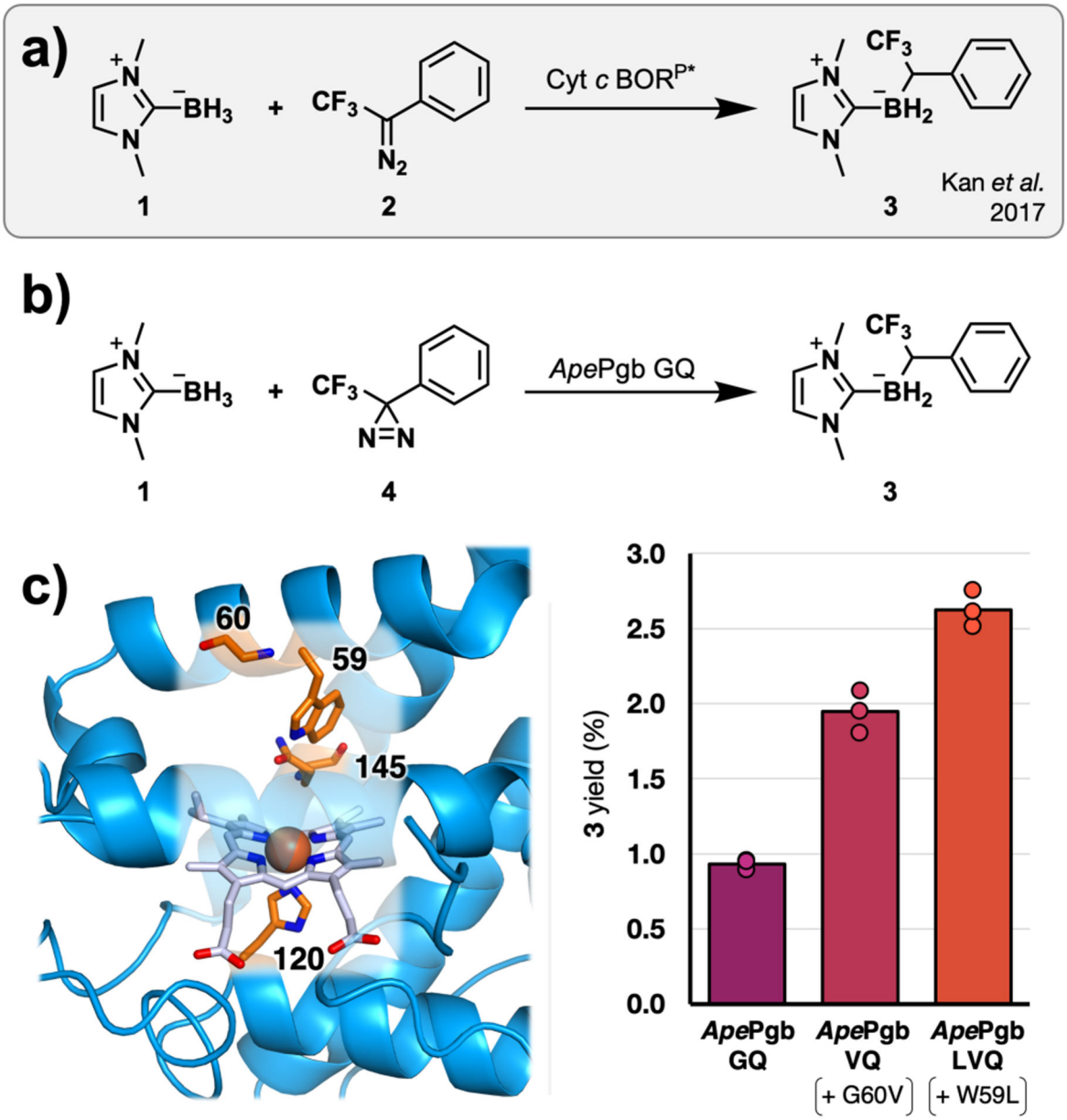


Figure 1.

(a) Previous biocatalytic B–H insertion reaction catalyzed by BOR^{P*}, a variant of *Rhodothermus marinus* cytochrome *c*. **(b)** Reaction targeted in enzyme discovery substitutes the diazo with the equivalent diazirine carbene source. c) Amino acid positions targeted in evolution (orange) shown on an *Ape*Pgb GQ homology model (blue) alongside evolved improvements in B–H insertion yield in whole cell reactions (OD_{600} = 60).

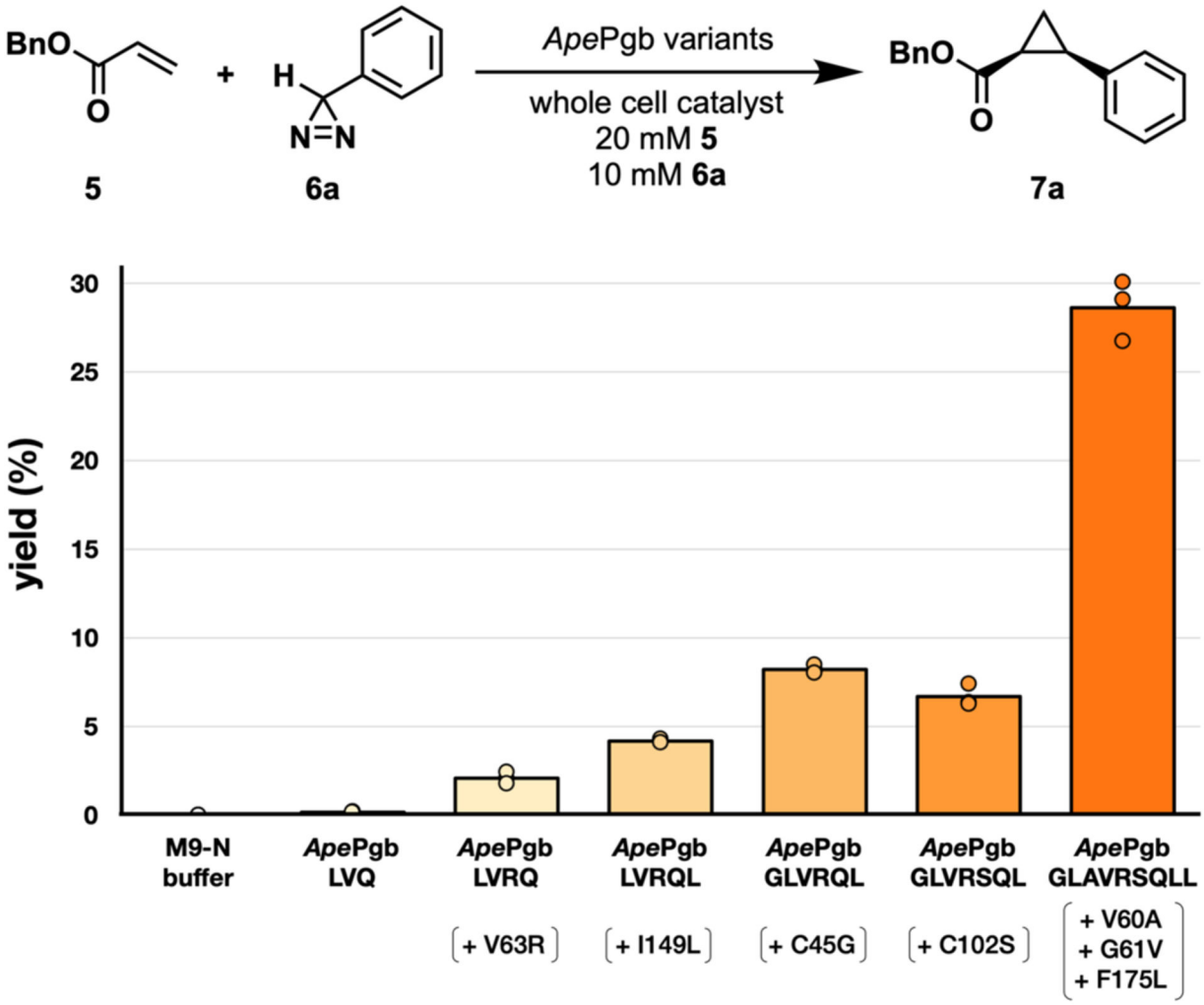


Figure 2.

Cyclopropanation reaction targeted in this work using *E. coli* cells harboring *Ape*Pgb variants (relative product stereochemistry shown) and yields for each sequentially evolved variant, showing a >150-fold improvement overall.

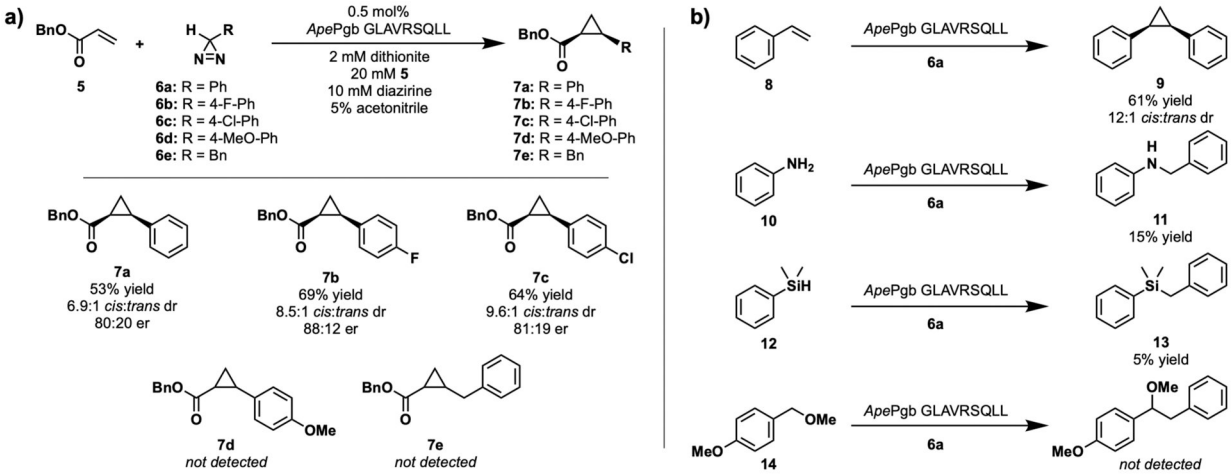


Figure 3.

(a) Enzyme-catalyzed cyclopropanation of benzyl acrylate with aryldiazirines. Reactions were performed anaerobically at room temperature using purified *Ape*Pgb GLAVRSQLL in 50 mM potassium phosphate (pH = 8.0) and 150 mM NaCl. Yields and diastereomeric ratios were quantified by GC-FID. Enantioselectivities were quantified by HPLC (7a) or SFC (7b & 7c) on a chiral stationary phase (details in SI). **(b)** Alternative carbene transfer reactions carried out using purified enzyme under identical conditions to those shown in (a), only substituting the shown substrate for benzyl acrylate. All yields and dr measurements were quantified by GC-FID.

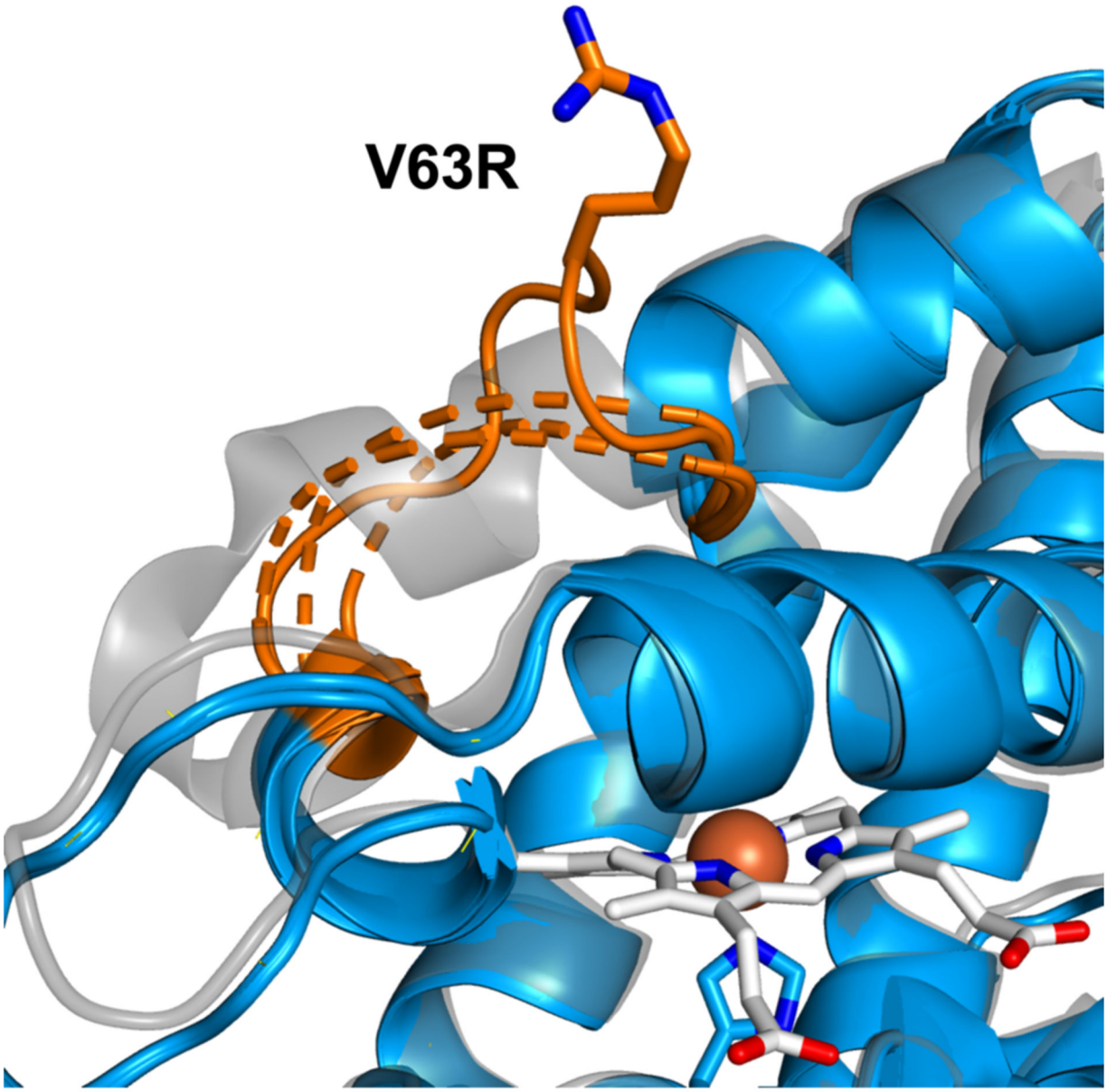
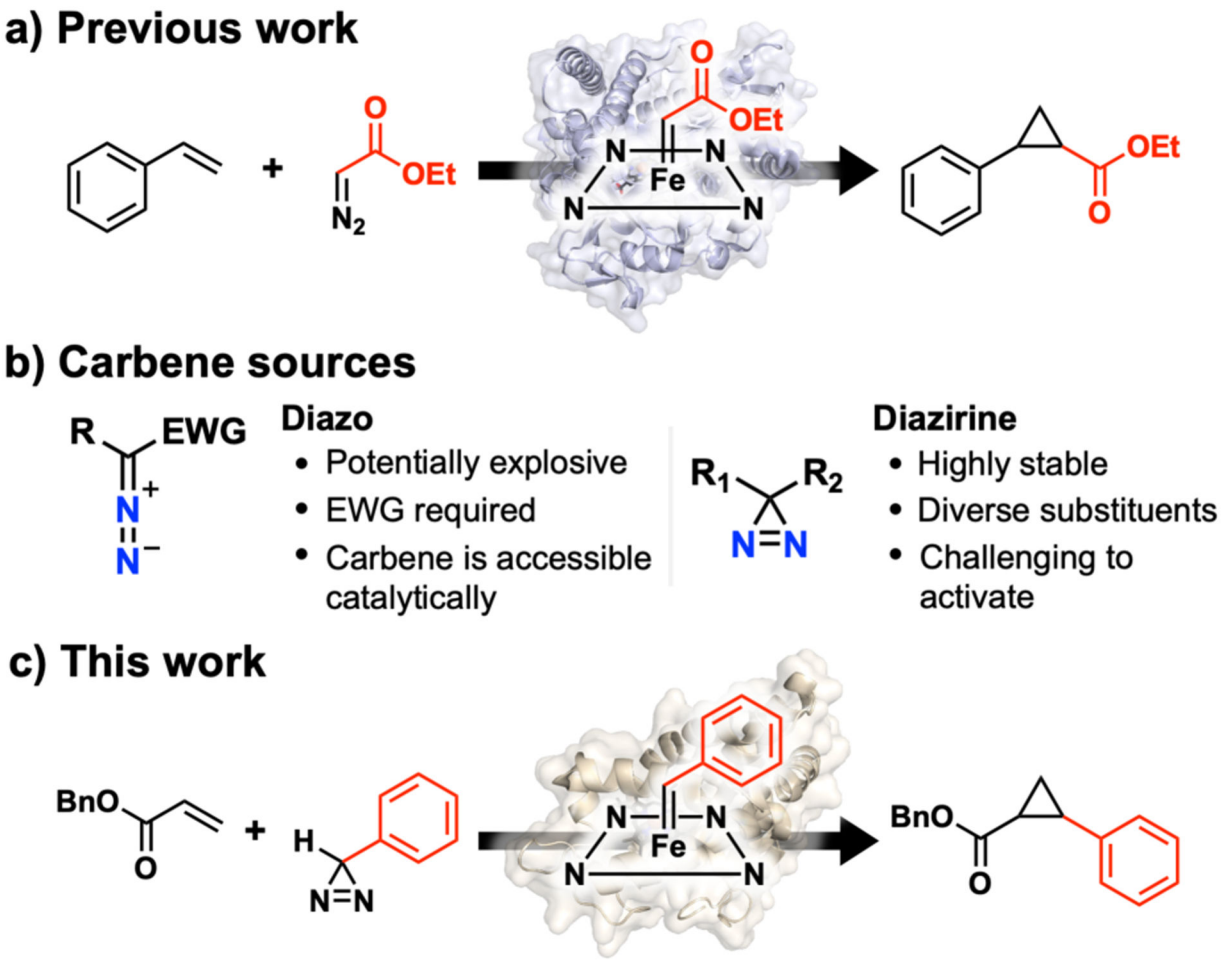


Figure 4.

Superposition of Y61A *M. acetovorans* protoglobin (gray; PDB 3ZJI) with the four monomers observed in the MicroED structure of *Ape*Pgb GLVRSQL (blue) with positions 60–70 in orange, showing the reconfigured conformation of these residues relative to the helical conformation in the *M. acetovorans* protoglobin (76 % sequence similarity). Dashed lines represent regions disordered in subunits B, C, and D of the unit cell in comparison with the resolved loop in subunit A.



Scheme 1.
Background and summary